TITLE OF THE INVENTION

[0001] Geldanamycin-producing strains, uses thereof and methods of producing same

FIELD OF THE INVENTION

[0002] The present invention relates to geldanamycin-producing strains, uses thereof and methods of producing same. More specifically, the present invention is concerned with these strains and uses thereof as biocontrol agents against common scab.

BACKGROUND OF THE INVENTION

[0003] Common scab is a disease widely distributed in potato-growing areas. Superficial or deep corky lesions on potato tubers characterise the disease. *Streptomyces scabies* (Lambert and Loria, 1989) is the main causal agent of the disease (Goyer et al., 1996). Methods used to control common scab include chemical treatments of seed potato tubers (Davis et al., 1976), irrigation (Adams and Lapwood, 1978), soil amendments (Weinhold and Brown, 1968), cultivar choice (Bouchek-Mechiche et al., 2000) and rotation strategies (Li et al., 1999).

[0004] Biological control of potato scab by nonpathogenic streptomycetes was also reported (Doumbou et al., 2001b; Doumbou et al., 1998; Liu et al., 1995a; Ryan and Kinkel, 1997). For example, introduction into an infested soil of some *Streptomyces diastatochromogenes* and *S. albogriseolus* strains isolated from suppressive soils decreased common scab symptoms on potato tubers (Eckwall and Schottel, 1997; Liu et al., 1995a; Lorang et al., 1995). Common scab severity was also decreased by an

amendment of an antibiotic biofertilizer produced from swine feces containing *S. albidoflavus* strain CH-33 (Hayashida et al., 1989). These strains are not known to produce geldanamycin.

[0005] Geldanamycin is known to inhibit the growth of several plant pathogenic fungi (Toussaint et al., 1997) and geldanamycin-producing streptomycetes were shown to protect crops against several fungal diseases (Rothrock and Gottlieb, 1984; Valois et al., 1996). This antibiotic is also active against some Gram-positive bacteria (Toussaint et al., 1997) such as *S. scabies* (Agbessi, 2002) but the efficiency of this bioagent to control common scab has not yet been determined. It cannot be predicted whether a strain known to be antagonistic against a microbiological pathogen will be able to compete in the rhizosphere against this pathogen and against other soil microbiological pathogens. It cannot therefore be predicted whether this strain would be effective in controlling a plant disease caused by this microbiological pathogen.

[0006] Certain references have shown that specific chitin waste-based composts suppress common scab (Côté et al., 2001; Vruggink (1970)). It was never demonstrated however whether the little amount of chitosan contained in these wastes participated in controlling the disease. Some success of biocontrol have been reported by the combined use of a biocontrol agent and of chitosan without however demonstrating the role played by chitosan itself (Cuero et al., 1991; Singh et al., 1999). Chitosan is known to be toxic against most microorganisms. There thus remains a need to demonstrate whether chitosan may help control common scab and whether it may be used in combination with microorganisms antagonistic to common scab.

[0007] There thus remains a need for new effective biocontrols of

common scab.

SUMMARY OF THE INVENTION

[0008] The present invention demonstrates for the first time the biocontrol efficiency of geldanamycin-producing strains against a bacterial plant disease and more particularly against common scab.

[0009] More specifically, in accordance with the present invention, there is provided a use of an inoculum of a geldanamycin-producing strain able to survive in a plant rhizosphere as a biocontrol of common scab affecting the plant. In a specific embodiment, the strain encodes a protein having the sequence of SEQ ID NO: 6 or a homologous sequence having geldanamycin activity. In an other embodiment, the strain comprises the nucleotide sequence of SEQ ID NO: 7 or a homologous sequence having geldanamycin activity. In an other embodiment, the strain comprises a nucleotide sequence able to hybridize under high stringency conditions to the complementary sequence of a sequence selected from the group consisting of : the nucleotide sequence of SEQ ID NO: 1, the nucleotide sequence of SEQ ID NO: 2, the nucleotide sequence of SEQ ID NO: 3, the nucleotide sequence of SEQ ID NO: 5, and the nucleotide sequence of SEQ ID NO: 7. According to specific embodiments, the strain is a *Streptomyces* strain. In more specific embodiments, the strain is selected from the group consisting of *Streptomyces violaceusniger*, *Streptomyces hygroscopicus* and *Streptomyces melanosporafasciens* strains. In more specific embodiments, the strain is deposited at the American Type Culture Collection (ATCC) under Accession number BAA-668. According to specific embodiment, the biocontrol comprises a reduction of the severity of common scab or a reduction of the incidence of common scab.

[0010] According to an other aspect of the invention, there is provided a biologically pure culture of a *Streptomyces* strain deposited at the American Type Culture Collection (ATCC) Accession number BAA-668, or a variant thereof.

[0011] According to an other aspect of the invention, there is provided a composition comprising an inoculum of a *Streptomyces* strain deposited at the American Type Culture Collection (ATCC) Accession number BAA-668 and a carrier. In a specific embodiment, the carrier comprises chitosan.

[0012] According to an other aspect of the invention, there is provided a method of biocontrolling common scab comprising applying on a plant an effective amount of an inoculum of a geldanamycin-producing strain able to survive in the plant rhizosphere. In a specific embodiment, the strain encodes a protein having the sequence of SEQ ID NO: 6 or an homologous sequence having geldanamycin activity. In an other embodiment, the strain comprises the nucleotide sequence of SEQ ID NO: 7 or a homologous sequence having geldanamycin activity. In an other embodiment, the strain comprises a nucleotide sequence able to hybridize under high stringency conditions to the complementary sequence of a sequence selected from the group consisting of : the nucleotide sequence of SEQ ID NO: 1, the nucleotide sequence of SEQ ID NO: 2, the nucleotide sequence of SEQ ID NO: 3, the nucleotide sequence of SEQ ID NO: 5, and the nucleotide sequence of SEQ ID NO: 7. According to specific embodiments, the strain is a *Streptomyces* strain. In more specific embodiments, the strain is selected from the group consisting of *Streptomyces violaceusniger*, *Streptomyces hygroscopicus* and *Streptomyces melanosporafasciens* strains. In more specific embodiments, the strain is deposited at the American Type Culture Collection (ATCC) under Accession number BAA-668. According to specific embodiment, the biocontrol comprises

a reduction of the severity of common scab or a reduction of the incidence of common scab.

[0013] According to an other aspect of the present invention, there is provided a method for modifying the biocontrol efficiency of a bacterial strain comprising intraspecific protoplasm fusion of the bacterial strain with an other strain having a desirable biocontrol property against common scab.

[0014] According to an other aspect of the present invention, there is provided a method for making a biocontrol agent against common scab, which comprises the steps of: obtaining a microbial strain susceptible of producing geldanamycin; contacting the nucleic acids or the proteins of said strain with a ligand specific to geldanamycin or to a nucleic acid encoding geldanamycin; detecting the formation of a complex as an indication of the presence of geldanamycin or of a nucleic acid encoding geldanamycin in the strain, whereby said strain or a geldanamycin-producing part of said strain may be used as a biocontrol agent against common scab. In a specific embodiment, the ligand is a nucleic acid having at least 12 nucleotides in length hybridizing with the nucleic acids having a sequence complementary to a sequence selected from: a) the nucleotide sequence of SEQ ID NO: 1; b) the nucleotide sequence of SEQ ID NO: 2; c) the nucleotide sequence of SEQ ID NO: 3; d) the nucleotide sequence of SEQ ID NO: 5; and the nucleotide sequence of SEQ ID NO: 7. The ligand may also bind to the geldanamycin protein. Such ligand may include an antibody specific to the geldanamycin.

[0015] Robinson et al. (1981) showed that it was possible to increase the level of antibiotic production by protoplast fusion. Biosynthesis of new compounds by recombinant strains obtained by intraspecific protoplast fusion (Fujimoto et al. 1990) as well as by interspecific protoplast fusion (Xiufen and

Qi 1989) has been previously reported. The present invention also therefore concerns a method for modifying the biocontrol efficiency of a *Streptomyces* strain comprising protoplast fusion. The Applicant was the first to use such method for modifying the biocontrol efficency of a microorganism.

[0016] As used herein, the terminology "geldanamycin-producing strain" refers to any bacterial strain producing geldanamycin including strains naturally found in the nature such as EF-76, *Streptomyces hygroscopicus* var. *geldanus* ATCC 55256, *Streptomyces violaceusniger* YCED9, *Streptomyces hygroscopicus* strain NRRL 3602. It also refers to any synthetic strain producing geldanamycin such as a recombinant strain produced according to means known by persons of ordinary skill in the art and strains obtained from protoplasm fusion including FP-54. These means include methods of cloning genes of a geldanamycin-producing strain into a non-producing strain.

[0017] The terminology "geldanamycin-producing strain" also includes strains containing a sequence encoding a protein identified in Rasher *et al.* "Cloning and characterization of a gene cluster for geldanamycin production in *Streptomyces hygroscopicus*" *Microbiol. Lett.* 218 (2), 223-230 (2003) as a secreted protein (SEQ ID NO : 6). This sequence is also available in NCBI database under no AY179507. It is reasonably predictable that this secreted protein is geldanamycin. The definition therefore also includes strains containing a nucleotide sequence encoding this protein, namely nucleotide sequence (SEQ ID NO : 7). The definition also includes strains producing a protein having this sequence (SEQ ID NO : 6) or an homologous protein having a geldanamycin activity. « Homologous » is intended to mean a protein similar or identical to geldanamycin which is produced by a variant *S. melanosporofasciens* strain, another *Streptomyces* species, another microbial species (e.g. fungus or bacteria) of a natural or synthetic origin. Such

homologous or corresponding protein shares amino acid and nucleotide sequences susceptible to encode a protein having the same activity profile as geldanamycin. This activity is monitored on sensitive strains like *S. scabies*. The definition also includes strains containing nucleotide sequences able to hybridize under stringent conditions to a sequence complementary to sequences involved in geldanamycin biosynthesis. For instance, FP-60, a strain shown not to produce geldanamycin does not hybridize to probes Bm27, Bm3 and BS15. This definition also includes strains possessing a gene encoding an amino DHQ synthase. Indeed, EF-76 (data not shown) and *Streptomyces hygroscopicus* strain NRRL 3602 (see NCBI AY179507) possess a gene encoding an amino DHQ involved in geldanamycin biosynthesis.

[0018] As used herein, the terms "EF-76" and "*Streptomyces melanosporofasciens*" are used interchangeably.

[0019] As used herein, the terminology "biologically pure" strain is intended to mean a strain separated from materials with which it is normally associated in nature. Note that a strain associated with other strains, or with compounds or materials that it is not normally found with in nature, is still defined as "biologically pure." A monoculture of a particular strain is, of course, "biologically pure."

[0020] For the methods and uses of the present invention, it is not necessary that the whole broth culture of the strains of the invention be used. Indeed, the present invention encompasses the use of a whole broth culture of a strain of the present invention, spores produced by such strain, dried biomass of the strains and lyophilized strains. As used herein therefore, the terminology "inoculum of a strain" refers to any form or part of the strain of the present invention or a combination thereof that possesses the desired ability to control

common scab.

[0021] There is also provided a combination of an inoculum of a strain according to the present invention and of a carrier.

[0022] In order to achieve good dispersion, adhesion and conservation/stability of compositions within the present invention, it may be advantageous to formulate the whole broth culture or supernatant with components that aid dispersion, adhesion and conservation/stability or even assist in the biocontrol of the plant disease. These components are referred to herein individually or collectively as "carrier". Suitable formulations for this carrier will be known to those skilled in the art (wettable powders, granules and the like, or carriers within which the inoculum can be microencapsulated in a suitable medium and the like, liquids such as aqueous flowables and aqueous suspensions, and emulsifiable concentrates). Other suitable formulations will be known to those skilled in the art. The carrier may include components such as chitosan, vermiculite, compost, talc, milk powder, gel, etc.

[0023] Chitosan, a chitin deacetylated derivative, is another product that was shown effective to control several fungal diseases (Benhamou and Thériault, 1992; Sathiyabama and Balasubramanian, 1998). Protection conferred to plants by chitosan depends on both the elicitation of plant defense mechanisms (Kauss et al., 1989; Pearce and Ride, 1982; Walker-Simmons et al., 1983) and the fungicidal property of chitosan oligomers (Allan and Hadwiger, 1979; Hirano and Nagao, 1989). Chitosan is known to exhibit bacteriostatic activity towards Gram-negative and Gram-positive human pathogens (Allan et al., 1984), foodborne pathogens (Wang, 1992) and lactic bacteria (Savard et al., 2002). Chitosan has usually no toxic effect on microorganisms producing chitosanases. Chitosanolytic organisms would

benefit from the presence of chitosan in their environment as carbon and nitrogen sources. Chitosanolytic activities have been reported for strains of different bacterial genera including *Streptomyces* (Fukamizo and Brzezinski, 1997).

[0024] As used herein, the terms "mutant" and "variant" are used interchangeably. A variant of the EF-76 strain deposited at the ATCC under access no BAA-668 may or may not have the same identifying biological characteristics of the EF-76 strain, as long as the variant possesses biocontrol efficiency against common scab. Illustrative examples of suitable methods for preparing variants of the inventive microorganism strain include, but are not limited to: intraspecific protoplast fusion, mutagenesis by irradiation with ultraviolet light or X-rays, or by treatment with a chemical mutagen such as nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine), methylmethane sulfonate, nitrogen mustard and the like; gene integration techniques, such as those mediated by insertional elements or transposons or by homologous recombination of transforming linear or circular DNA molecules; and transduction mediated by bacteriophages such as P1. These methods are well known in the art and are described, for example, in J. H. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1972); J. H. Miller, A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1992); M. Singer and P. Berg, Genes & Genomes, University Science Books, Mill Valley, CA (1991); J. Sambrook, E. F. Fritsch and T. Maniatis, Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); P. B. Kaufman *et al.,* Handbook of Molecular and Cellular Methods in Biology and Medicine, CRC Press, Boca Raton, FL (1995); Methods in Plant Molecular Biology and Biotechnology, B. R. Glick and J. E. Thompson, eds., CRC Press, Boca Raton, FL (1993); and P. F. Smith-Keary, Molecular Genetics

of *Escherichia coli*, The Guilford Press, New York, N.Y. (1989).

[0025] Variant strains derived from the EF-76 strain using known methods are then preferably selected or screened for biocontrol efficiency against common scab. In a specific embodiment, fusant cells are selected on the basis of their ability to control common scab. Strain FP-54 described herein constitutes a variant as defined herein. It is a strain obtained from the protoplasm fusion of EF-76 and possesses biocontrol efficiency against common scab.

[0026] As used herein, the terminology "biocontrol" is meant herein to refer to reduction of severity or reduction of incidence a plant disease.

[0027] As used herein, the terminology "biocontrol efficiency" of a strain refers to the strain's ability to ameliorate or stabilize the state of a plant affected by a disease or reverse, slow or delay progression of the plant disease. It may be assessed though a number of parameters including: antibiotic production, decrease of the plant disease severity index, antagonistic activity against a plant pathogen, antibiosis, lysis, phytotoxin degradation and rhizocompetence. When the biocontrol efficiency assessed is that of strains of the present invention against common scab, the parameters used include antagonistic activity against *S. scabies*, geldanamycin production, disease severity index of common scab, disease incidence of common scab. As used herein, the term "disease incidence" refers to the percentage of tubers or roots displaying visible signs of common scab lesions.

[0028] As used herein, the terminology "common scab" is meant to include any *Streptomyces*-induced common scab including those affecting root and tuber food crops such as red and sugar beets, carrots, parsnips, radishes,

rutabagas and turnips (Goyer et al. 1997). Geldanamycin was shown to be active against other common scab causing bacterial species including *acidiscabies* and *caviscabies* (data not shown).

[0029] As used herein, the terminology "severity of common scab" is meant to refer to the scope of common scab symptoms on vegetal tissues. It may be assessed through a number of means including the determination of the extent of the surface of the plant covered by scab lesions.

[0030] As used herein, the terminology "effective amount" is meant to refer to an amount sufficient to effect beneficial or desired results. An effective amount can be provided in one or more administrations. In terms of treatment of and protection against common scab, an "effective amount" is an amount sufficient to ameliorate, stabilize, reverse, slow or delay progression of the plant disease state. In specific embodiments, an "effective amount" is an amount sufficient to ameliorate, stabilize, reverse, slow or delay progression of the plant disease state by at least 5% with regard to a non-treated diseased control plant In other specific embodiments, the effective amount may be comprised between about 10^3 and 10^9 spores/g of carrier.

[0031] According to the methods of the present invention for applying the inoculum of a strain according to the present invention, the inoculum may be applied on various parts of the plant affected by common scab including the tubers and the root and any part thereof. It may be applied at plantation or later during the season.

[0032] Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments thereof, given by way of example only

with reference to the accompanying drawings.

[0033] The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

[0034] In the appended drawings:

[0035] Figure 1 shows the growth of *Streptomyces scabies* EF-35 in the presence of *Streptomyces melanosporofaciens* strains FP-60 (A), EF- 76 (B), and FP-54 (C);

[0036] Figure 2 presents a southern blot hybridization between BamHI-digested genomic DNAs of *S. melanosporofaciens* strains EF-76, FP-54 and FP-60 and probe Kp38 (SEQ ID NO: 1);

[0037] Figure 3 presents a southern blot hybridization of Bam- HI-digested genomic DNAs and probes Bm27 (SEQ ID NO: 2) (A), Bm3 (SEQ ID NO: 3) (B), and BS15 (C);

[0038] Figure 4 presents the effect of *S. melanosporofaciens* EF-76 and of two fusant strains on common scab of potato. Panel (A) and B) presents assays in a growth chamber and in the field, respectively. Bars accompanied by the same letter indicate that the corresponding treatments did not significantly differ ($p<0.05$) *;

[0039] Figure 5 shows the growth of *S. scabies* strain EF-35 (left) and of *S. melanosporofaciens* strain EF-76 (right) on a chitosanase detection medium;

[0040] Figure 6 shows the DNA sequence of probe Kp38 containing type-I polyketide synthase (PKS) genes (SEQ ID NO: 1);

[0041] Figure 7 shows the DNA sequence of probe Bm27 (SEQ ID NO: 2);

[0042] Figure 8 shows the DNA sequence of Bm3 (SEQ ID NO: 3);

[0043] Figure 9 shows the amino acid sequence of the putative aminoDHQ synthase of *Streptomyces hygroscopicus* strain NRRL 3602 (SEQ ID NO: 4);

[0044] Figure 10 shows the DNA sequence encoding the putative aminoDHQ synthase of *Streptomyces hygroscopicus* strain NRRL 3602 (SEQ ID NO: 5);

[0045] Figure 11 shows the amino acid sequence of the putative geldanamycin protein of *Streptomyces hygroscopicus* strain NRRL 3602 (SEQ ID NO: 6); and

[0046] Figure 12 shows the DNA sequence encoding putative geldanamycin protein of *Streptomyces hygroscopicus* strain NRRL 3602 (SEQ ID NO: 7);

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

EXAMPLE 1

Strains used

[0047] *Streptomyces scabies* strain EF-35 and *S. melanosporofaciens* strain EF-76 were isolated from common scab lesions on potato tubers (Faucher et al., 1992). *Streptomyces melanosporofaciens* EF-76 (Doumbou et al., 2001a), formely *S. hygroscopicus* sbsp. *geldanus*, was selected after screening for the ability to inhibit *Phytophthora fragariae* growth causing raspberry root rot (Valois et al., 1996). Strain EF-76 produces geldanamycin (Toussaint et al., 1997), a polyketide exhibiting antimicrobial activity (DeBoer et al., 1970).

EXAMPLE 2

Detection medium for chitosanase activity

[0048] Chitosanase detection medium was prepared according to Brzezinski et al. (1997) with the following modifications. The detection medium was prepared by adding successively to a sterile base medium (1.74 g of peptone and 3.6 g of agar in 170 ml of distilled water) 90 mL of chitosan solution, 30 mL of solution A, 10 mL of 0.25 M K_2HPO_4 and 1 mL of 5N KOH. Chitosan solution was prepared by dissolving 1 g of chitosan (Sigma-Aldrich, St-Louis, MO) in 100 mL of 0.1 N HCl. Solution A consisted of 10 g L-1 $MgSO_4$, 10 g L-1 NaCl, 1 g L-1 K_2HPO_4, 100 mg L-1 $FeSO_4$, 100 mg L-1 $CaCl_2$, 66 mg L-1 $MnCl_2$ and 7 mg L-1 $ZnCl_2$. Strains were inoculated on the chitosanase detection medium and incubated at 30 .C for 24 – 48 h. Chitosanase activity was detected by the formation of a clear zone of degradation around the growing colonies.

[0049] Figure 5 showed that strain EF-76 had the property to hydrolyze chitosan. A clearing zone was observed around EF-76 colony indicating that the insoluble chitosan contained in the growth medium was hydrolyzed. *S. scabies* strain EF-35 produced melanoid pigment on this medium but no chitosanase activity was detected. Treatments of seed pieces did not appear to affect the yield at harvest. Each year, the yield values of the four treatments did

not significantly differ ($P < 0.05$) (Table 2).

EXAMPLE 3

Greenhouse assay

[0050] The pathogenicity test was carried out using potato tubers (*Solanum tuberosum* cv. *Shepody*) obtained from glasshouse grown plants derived from tissue culture with no visible sign of disease. Potato tubers were planted in 250-mm diameter pots containing sand and vermiculite (2:1). *S. scabies* inoculum was prepared as previously described by Faucher et al. (1992). *S. scabies* strain EF-35 was grown for 2 weeks at 30 °C, in 50 mL tubes containing 3 g vermiculite saturated with Say-solution (Labruyère, 1971). This inoculum was mixed with the plant growth substrate before planting. At planting, talc or chitosan (0.5 g) with or without *S. melanosporofaciens* strain EF-76 was sprinkled on the top of each tuber. Spores were previously collected from a 10-day-old culture on YME agar and then mixed with talc or chitosan (10^8 spores/g of carrier). The pathogenicity test was carried out in five replicates. Pots were randomly dispersed in a greenhouse and their contents were watered every three days. A soluble fertilizer (20-20-20) was added every 2 weeks. The progeny tubers were harvested after three months and were then examined for common scab (Goyer et al., 1998). Disease severity was visually estimated by attributing a disease severity index (1 – 10) to each tuber in regard of the extent of their surfaces covered with scab lesions. A disease index of 1 was associated to healthy tubers while a disease index of 10 represented tubers covered by scab lesions on more than 90% of their surface.

[0051] The highest common scab incidence (43%) was associated with the talc only treatment. When compared to talc only, chitosan only reduced common scab incidence from 43 to 27% but this difference was not statistically different ($P < 0.05$). When progeny tubers were grown in the presence of *S.*

melanosporofaciens strain EF-76 independently of the carrier used (talc or chitosan), disease incidence was significantly reduced as compared to that obtained to the talc only treatment and was reduced, although not significantly, as compared to that obtained with the chitosan only treatment (Table 1).

Table 1. Effect of chitosan and *S. melanosporofaciens* EF-76 on common scab incidence under controlled conditions

Treatment	Common scab incidence (%)
Talc (control)	43a[1]
Chitosan	27ab
Spores of *S. melanosporofaciens* EF-76 in talc	11b
Spores of *S. melanosporofaciens* EF-76 in chitosan	14b

[1]Numbers accompanied by the same letters are not significantly different ($P < 0.05$, +2 test).

EXAMPLE 4

Field assay

[0052] Trials were performed in a naturally infested field at L'Assomption (Quebec, Canada) in 2000 and in 2001. At planting, a formulation powder (0.5 g) was added on the top of each tuber (*Solanum tuberosum* cv. *Shepody*). Formulations consisted of one of two carriers (talc or chitosan) with or without dried biomass of *S. melanosporofaciens* EF-76 (1/300 w:w inoculum/carrier). EF-76 biomass was prepared as follows. The bacteria were grown 7 – 10 days in a 5 L reactor, in YME liquid medium supplemented with 10 mM $CaCO_3$. The cultures were centrifuged and the pellets were freeze-dried for 18 h. An experimental plot consisted of 4 rows planted with 26 seed tubers. The plots were arranged as randomized complete blocks with 4 replicates. Common scab symptoms were estimated visually on 100 tubers harvested from each plot. The disease incidence as well as the disease severity was scored for each treatment. A disease severity index (1 – 10) was attributed to each tuber in regard of the extent of their surfaces covered with scab lesions. A disease index of 1 was associated to healthy tubers while a disease index of 10 represented tubers covered by scab lesions on more than 90% of their surface. Yield was determined by the weight (kg) of progeny tubers harvested from each plot.

[0053] The four treatments [talc (control treatment), chitosan, talc supplemented with *S. melanosporofaciens* spores, and chitosan supplemented with *S. melanosporofaciens* spores] were compared. Treatments of seed pieces did not appear to affect the yield at harvest. Each year, the yield values of the four treatments did not significantly differ ($P< 0.05$) (Table 2).

[0054] Common scab was more severe in 2000 than in 2001 as shown by both a higher average disease severity index (an average severity index of 2.43 for 2000 versus 1.43 for 2001) and a higher level of disease incidence (an average disease incidence of 80.3 for 2000 versus 35.8% for 2000) as seen in Table 2.

[0055] Each year, the highest common scab disease severity index was associated with the talc only treatment. Common scab severity was significantly lower for all other treatments except for the chitosan treatment in 2001. The level of protection against the disease incidence conferred by chitosan and *S. melanosporofaciens* spores into talc was equivalent since the disease severity index value for both treatments did not significantly differ in 2000.

[0056] If most treatments reduced common scab severity when compared to the talc treatment, only some treatments significantly reduced common scab incidence. In 2000, chitosan with or without *S. melanosporofaciens* spores reduced the disease incidence of 35 and 8%, respectively. In 2001, *S. melanosporofaciens* spores in talc or in chitosan reduced the disease incidence of 21 and 23%, respectively. The combination of chitosan and of *S. melanosporofaciens* spores offered in 2000 a protective effect against common scab that was higher than those conferred by chitosan or by *S. melanosporofaciens* spores in talc. This additive effect was not observed in 2001.

Table 2. Effect of chitosan and of *S. melanosporofaciens* EF-76 on common scab of potato under field conditions

Treatment	Field assay 2000			Field assay 2001		
	Disease severity index	Disease incidence (%)	Yield (kg/plot)	Disease severity index	Disease incidence	Yield (kg/plot)
Talc (control)	2.83a[1].	91a[2]	53.6a[1]	1.61a[1]	47a[2]	61.9a[1]
Chitosan	2.36b	83b	45.8a	1.53a	46a	60.6a
Strain EF-76 in talc	2.49b	91a	49.3a	1.26b	26b	58.8a
Strain EF-76 in chitosan	2.05c	56c	49.2a	1.31b	24b	56.6a

1 Numbers of the column that are accompanied by a same letter did not significantly differ (P <0.05, ANOVA test).
2 Numbers of the column that are accompanied by a same letter did not significantly differ (P <0.05, +2 test).

Effect of a combination of EF-76 and chitosan

[0057] EF-76 appears to be an efficient biocontrol agent against common scab of potato. For the two consecutive years in the field trial, this strain, when applied with talc, reduced disease severity as compared to that obtained with to the control treatment. EF-76 also reduced disease incidence in the 2001 field assay but not in 2000. The level of pathogenic inoculum possibly affected the ability of EF-76 to reduce common scab incidence. The disease incidence was reduced when the pathogen inoculum was moderate (the greenhouse assay and the 2001 field trial) but not when the inoculum was high (the 2000 field assay). Inoculation of potato seeds with EF-76 reduced the common scab index both in growth-chamber and field conditions.

[0058] The present invention presents the first demonstration of biocontrol by a geldanamycin-producing strain against a bacterial disease.

Effect of a combination of EF-76 and chitosan

[0059] As may be seen in Figure 1, EF-76 exhibited chitosanolytic activity and thus could be protected against the toxic effect of chitosan.

[0060] Combination of chitosan and EF-76 ensured in both controlled and field conditions a significant decrease of disease severity and of disease incidence when compared to the control treatment. Even better, the combined

use of strain EF-76 and of chitosan provided in some cases a higher protection than did strain EF-76 in talc or chitosan by itself. Even though this additive effect was not always observed, combined application of chitosan and EF-76 was always one of the best treatments. As the effectiveness of chitosan and of EF-76 in the control of common scab seems to be differentially influenced by environmental conditions such as the level of pathogen inoculum, the combined use of both products would ensure in most conditions a significant level of protection.

EXAMPLE 5

Intraspecific Protoplastic fusion of the wild-type strain EF-76

[0061] Protoplasts of the strain EF-76 were made according to the procedure described by Hopwood et al. (1985). A solution of 50% PEG 1000 in P buffer (Hopwood et al. 1985) was added to 10^8 protoplasts. These protoplasts were resuspended and kept 5 min at room temperature. Samples of this suspension were plated on R2YE medium (Hopwood et al. 1985) and incubated at 30°C. After their regeneration on R2YE plates, colonies were streaked on YME medium for the screening of strains improved or deficient in antibiotic production.

[0062] Several hundreds colonies were recovered from the protoplast fusion experiment. Among these colonies, 100 were streaked on YME medium and tested for their ability to inhibit the growth of *B. cereus* ATCC 14579. Seven isolates were positively or negatively affected in their inhibition power against *Bacillus* when compared to the wild-type strain EF-76.

[0063] Two fusants were studied in more detail, namely FP-54 (ATCC BAA-669) and FP-60 (ATCC BAA-670).

Prototrophy of FP-54 and FP-60

[0064] The nutritional requirements of strains FP-54 and FP-60 was verified by inoculating them on minimal medium [$(NH_4)_2 SO_4$ 2 g/l, K_2HPO_4 0.5 g/l, $MgSO_4 \cdot 7 H_2O$ 0.2 g/l, $FeSO_4 \cdot 7 H_2O$ 0.01 g/l, glucose 5 g/l and agarose 15 g/l]. Bacterial growth was observed after 5 days of incubation at 30 °C. The ability of the two fusant strains to produce b-1,3-glucanase, b-1,4-glucanase and b-1,6-glucanase was tested in the same minimal medium containing, instead of glucose, 0.4% laminarin (Sigma-Aldrich, St. Louis, Mo., USA), 1% carboxymethylcellulose (Fluka, Buchs, Switzerland) and 1% pustulan (Calbiochem, San Diego, Calif., USA), respectively. The plates were incubated 5 days at 30°C and then overlaid with a solution of Congo red (0.2%) for 10 min followed by two washes with a 1 M NaCl solution. A clear zone appeared around colonies when the carbohydrate had been degraded.

[0065] Like the wild-type strain EF-76, strains FP-54 and FP-60 were able to grow on a minimal medium. These strains retained the ability to catabolize laminarin, cellulose, and pustulan. Their growth patterns were different from that of the wild-type strain. At stationary phase, the biomass reached by strains FP-54 and FP-60 was smaller than that of strain EF-76 (data not shown).

Growth curves of EF-76, FP-54 and FP-60

[0066] Growth curves of strains EF-76, FP-54 and FP-60 were established as follows. Two-day-old cultures were used after standardisation to inoculate 100 ml of a fresh YME medium. The flasks were incubated at 30 °C. Ten-ml samples were then withdrawn periodically and the dry weight of cells recovered from these samples was determined. This experiment was carried out in triplicate.

Southern blot analysis

[0067] BamHI-digested genomic DNA was transferred onto Hybond N™ nylon membrane (Amersham Pharmacia Biotech, Baie d'Urfé, Canada). Genomic DNAs were hybridized with four probes (Kp38, Bm27, Bm3, BS15). These probes all came from a contiguous 40-kb DNA fragment of strain EF-76 genome (Agbessi 2002). Probes were labeled with digoxigenin-dUTP using a kit (Roche Molecular Biochemicals, Laval, Canada). The samples were prehybridized (150 min) and hybridized (overnight) at 68 °C. After the incubation, the membranes were washed twice in 2x SSC (150 mM NaCl, 15 mM sodium citrate) containing 0.1% SDS for 7 min at 68 °C and twice in 0.1x SSC containing 0.1% SDS for 20 min at 68 °C. As used herein, these conditions qualify as "high stringency conditions". Blots were developed with a colorimetric development kit according to manufacturer specifications (Roche Molecular Biochemicals).

Genetic characterization of strains FP-54 and FP-60

[0068] The taxonomic identity of two selected fusant strains (FP-54 and FP-60) was determined by partial sequencing of the 16S rRNA gene (Doumbou et al. 2001a).

[0069] Strains FP-54 and FP-60 exhibited a 16S rDNA sequence identical to that of strain EF-76 over the 600-bp region sequenced for the three strains. Probes Kp38, Bm27, Bm3 and BS15 were hybridized with the genomic DNA of strains FP-54 and FP-60 to detect genetic modifications.

[0070] Figure 2 presents the hybridization between BamHI-digested genomic DNAs of *S. melanosporofaciens* strains EF-76, FP-54 and FP-60 and probe Kp38. This probe contains regions of the ketoacylsynthase and acyltransferase domains of type-I polyketide synthase (PKS) genes (SEQ ID

NO: 1). Since type-I PKSs multifunctional enzymes have repetitive domains, hybridization between *S. melanosporofaciens* genomic DNA and probe Kp38 gives several signals. The two most intense signals that correspond to the 3.8 and 5.5 BamHI fragments of strain EF-76 were absent from the hybridization pattern of strain FP-60.

[0071] Figure 3 presents a southern blot hybridization of Bam- HI-digested genomic DNAs of *S. melanosporofaciens* strains EF-76, FP-54 and FP-60 and probes Bm27 (A), Bm3 (B), and BS15 (C). Probe Bm27 contains a sequence encoding a putative formaldehyde dehydrogenase. Probe Bm3 contains some sequences encoding a putative transporter, a chitosanase, and a glycosyl transferase. Probe BS15 contains some sequences encoding a putative alcohol dehydrogenase and a methyl malonate- semialdehyde dehydrogenase.

[0072] No modification was detected in the genome of strain FP-54. The hybridization pattern of strain FP-60 with probe Kp38 was different from those of strains EF-76 and FP 54. Strain FP-60 was the only strain displaying no hybridization signal with probes Bm3, Bm27 and BS15.

EXAMPLE 6

In vitro antibiosis assays

Crossed resistance between EF-76, FP-54 and FP-60

[0073] Crossed resistance tests between strain EF-76 and FP-54 and FP-60 strains were carried out as described above except that YME was used instead of TSA and the plates were incubated 48 h following the overlay. Each of the three strains was individually plated on YME and their antagonistic properties against the two other strains were recorded.

[0074] FP-60's growth was inhibited in the presence of strains EF-76 and FP-54 (data not shown). Growth of strains FP-54 and EF-76 was not affected by the presence of each other or by the presence of strain FP-60 (data not shown). Since a strain that produces an antibiotic is generally resistant to it and one that is sensitive to this antibiotic does not produce it, these results suggest that FP-60 does not produce geldanamycin while EF-76 and FP-54 do.

Antibiosis of EF-76 against *B. cereus* and *S. scabies*

[0075] The ability of *S. melanosporofaciens* strains to inhibit the growth of *B. cereus* ATCC 14579 and *S. scabies* EF-35 was tested on YME as follows. *S. melanosporofaciens* strains (10^8 spores) were streaked in the center of YME plates. The plates were incubated 5 days at 30°C and then covered with an overlay of soft TSA (0.3% agar) containing *B. cereus* ATCC 14579 or *S. scabies* EF-35 (500 µl of an overnight culture in 4 ml of soft TSA). After 24 h at 30 °C, the diameter of the antibiosis zones around the Streptomyces inoculum was recorded. This experiment was carried out in five replicates. Strain EF-76 was shown to inhibit the growth of *S. scabies*.

Antibiosis of FP-54 and FP-60 against *P. fragariae*, *Bacillus cereus*, and *S. scabies* EF-35

[0076] The antagonistic property of the fusants was tested on *Bacillus cereus* ATCC 14579, *Phytophthora fragariae* var. *rubi* 390 and *S. scabies* EF-35. The *Streptomyces* strains, *B. cereus* and *P. fragariae* var. *rubi* were grown on yeast malt extract (YME) broth (4 g L-1yeast extract, 4 g L-1 glucose, 10 g L-1 malt extract, 15 g L-1 agar) or agar (15 g/l) (Pridham et al. 1956–1957), on trypticase soy broth (TSB), and on potato dextrose agar (PDA) (Difco Laboratories, Montreal, Canada), respectively.

[0077] The ability of fusants to inhibit the growth of *B. cereus* ATCC 14579

and of *S. scabies* EF-35 was tested on YME plates as follows. *Streptomyces* strains (10^8 spores) were streaked in the center of YME plates. The plates were incubated 5 days at 30°C and were then covered with an overlay of soft trypticase soy agar medium (TSA, 0.3 % agar) containing *B. cereus* ATCC 14579 or *S. scabies* EF-35 (500 µl of an overnight culture in 4 ml of inoculum soft TSA). After 24 h at 30°C, the diameter of the antibiosis zones around the *Streptomyces* inoculum was recorded. This experiment was carried out in five replicates.

[0078] The ability of the fusant strains to inhibit *P. fragariae* var. *rubi* was tested as follows. Fusant strains were streaked (10^8 spores) in the center of PDA plates and incubated for 2 days at 30 °C. A piece of PDA medium (8-mm diameter) from a 7-day-old culture of *P. fragariae* var. *rubi* 390 was then placed 1 cm from the border of the *Streptomyces* inoculum. *Phytophthora* growth inhibition was recorded after 5–7 days of incubation at 15°C.

[0079] Table 1 below shows the growth inhibition achieved by EF-76, FP-54 and FP-60 against certain strains and their retardation factor (Rf), namely the ratio of the compound migration distance on distance traveled by the solvent front. As may be seen in Table 1, strain FP-60 lost the ability to inhibit the growth of *B. cereus* ATCC 14579, *P. fragariae* var. *rubi* 390 and *S. scabies* EF-35 (Fig. 1). Strain FP-54 exhibited higher antagonistic activities, defined herein as the growth inhibition zone, against these three microorganisms than did the wildtype strain EF-76 (Fig. 1). The higher antagonistic property of strain FP-54 might be the consequence of a cumulative effect of various secondary metabolites. Indeed, strain FP-54 was shown to produce, in addition to geldanamycin, two other antimicrobial compounds that were absent in culture supernatants of strain EF-76.

Table 1 Antagonistic properties of EF-76 and fusant strains FP-54 and FP-60. _ No growth inhibition, + growth inhibition, ++ higher level of growth inhibition

Strains	Antagonistic properties against *Bacillus cereus*, *Streptomyces scabies* and *Phytophtora fragariae* var. *rubi*[a]	Retardation factor (Rf) of products inhibiting the growth of *Bacillus cereus*[a]
EF-76	+	0.51[b], 0.44
FP-54	++	0.85, 0.72, 0.51[b], 0.44
FP-60	_	no compound found

[a] Compounds were separated by thin layer chromatography on silica gel 60F-250
[b] Rf corresponding to geldanamycin (Toussaint et al. 1997)

Antibiosis of supernatant

[0080] Antibiotics were isolated from 96-h-old YME cultures. Culture supernatants (500 ml) were filtered through paper (Osmonics™, Minnetonka, Minn., USA) and the filtrates were extracted three times with one-third volume of chloroform. The chloroform fractions were evaporated on a BüChi Rotavapor R-14™ (Büchi Laboratoriums, Flawil, Switzerland). The resulting material was then dissolved in chloroform and separated by thin-layer chromatography on glass plates precoated with 0.5 mm silica gel 60F-250 using chloroform:methanol (95:5, v/v). After migration, the dried TLC was overlaid with soft TSA containing *B. cereus* ATCC 14579. The TLC plate was then incubated overnight at 30°C and the presence of growth inhibition zones was recorded.

[0081] In addition to geldanamycin, strain FP-54 produced antibiotics that were absent in strain EF-76 supernatant (Table 1).

[0082] Strain FP-60 lost the ability to synthesize geldanamycin (Table 1).

[0083] This example shows that intraspecific protoplast fusion can be

used to modify the biocontrol agent's efficiency of the strains of the present invention.

EXAMPLE 9

Effect on potato scab of seed inoculation with two fusants of EF-76

[0084] The ability of the fusant strains to reduce common scab symptoms was tested both in controlled and field conditions.

Growth chamber

[0085] Inoculum for the growth chamber assay were prepared by growing *S. scabies* EF-35 for 2 weeks at 30 °C in 50-ml tubes containing vermiculite saturated with Say-solution (Faucher et al. 1992). The antagonistic strain EF-76 and the fusant strains FP-54 and FP-60 were grown on YME agar for 10 days at 30 °C. Their spores were collected with a glass beads and then mixed with talc (10^8 spores/g talc). Scab-free potato tubers cv. *Green Mountain* were planted in 25-cm-diameter pots containing sterile sand and vermiculite (2:1, w/w) mixed with the pathogenic inoculum. At plantation, 0.5 g of talc with or without an actinomycete strain was sprinkled on the top of each tuber. Potatoes were grown at 25 °C with a 16-h photoperiod. Progeny tubers were harvested after 12 weeks and examined visually for common scab symptoms. A disease index (1–10) corresponding to the surface of coverage by common scab lesions was assigned to each infected tuber. On this scale, 1 means no disease while 10 means 100% surface coverage. The experiment was carried out in five replicates.

Field

[0086] The field experiment was carried out in a field naturally infested by *S. scabies* (L'Assomption, Canada). Plots consisting of four rows of 0.5m x 4m were planted with 104 potato seeds cv. *Shepody*. Plots were arranged as a

completely randomized block with four replicates. The inoculum to be applied on the tubers was prepared as follows. Strains EF-76, FP-54 and FP-60 were grown in YME supplemented with 10 mM $CaCO_3$ for 7–10 days in a 5-l bioreactor. The cultures were centrifuged and the pellets were freeze-dried for 18 h. Dried biomass (1 g) prepared as described before? was mixed with talc (300 g). Talc containing lyophilized bacteria (dried biomass) (0.5 g) was sprinkled on the top of each tuber at plantation. In control plots, talc without bacteria was applied on potato seeds. At harvest, tubers were examined for common scab symptoms as described above.

[0087] *S. melanosporofaciens* EF-76 had the capacity to reduce common scab both in controlled and field conditions. The disease index was reduced from 6.30 to 4.81 and from 2.83 to 2.49 in growth chamber and field experiments, respectively (Fig. 4). Strain FP-54 also reduced common scab severity on potato tuber, but no significant difference was observed between the disease index attributed to tubers treated with strain EF-76 or with strain FP-54 (Fig. 4).

[0088] It was observed that FP-54 strain survived in soil and significantly reduced the common scab disease incidence as compared to the reduction achieved with the control and EF-76. The disease incidence of tubers treated with FP-54 was of 56%, that with those treated with the control was of 72%, and that those treated with EF-76 was of 70%.

[0089] Strain FP-60 showed no protective effect against common scab; moreover, the disease index of tubers treated with this recombinant was higher than the index associated with potato tubers from the control treatment (Fig. 4). This suggests that geldanamycin biosynthesis is a mechanism associated with biocontrol. Strain FP-60 is not only ineffective as a biocontrol tool but potatoes

treated with this fusant had a disease index higher than potatoes from control treatment.

[0090] It also appears that the strains capable of producing geldanamycin, for instance strains EF-76 and FP-54, are effective against the scab causative agent.

[0091] It has been shown in Example 5 under subtitle "Genetic characterization of strains FP-54 and FP-60" that EF-76 and FP-54 possess sequences homologous to probes Bm3, Bm27 and BS15 while FP-60 does not. These results therefore suggest that the production of geldanamycin by EF-76 and FP-54 coincides with the presence of each or all sequences homologous to probes Bm3, Bm27 and BS15 in these strains.

EXAMPLE 6

***In vitro* antibiosis assays with other geldanamycin-producing strain**

[0092] The ability of *Streptomyces hygroscopicus* var. *geldanus* ATCC 55256 to inhibit the growth of *B. cereus* ATCC 14579 and of *S. scabies* EF-35 was tested on YME plates as follows. *Streptomyces* strains (10^8 spores) were streaked in the centre of YME plates. The plates were incubated 5 days at 30°C and were then covered with an overlay of soft trypticase soy agar medium (TSA, 0.3 % agar) containing *B. cereus* ATCC 14579 or *S. scabies* EF-35 (500 µl of an overnight culture in 4 ml of inoculum soft TSA). After 24 h at 30°C, the diameter of the antibiosis zones around the *Streptomyces hygroscopicus* var. *geldanus* inoculum was recorded. This experiment was carried out in three replicates. The antibiosis capacity of ATCC 5256 was similar to that of EF-76.

[0093] It is therefore submitted that geldanamycin-producing strains may be used as biocontrol agents against common scab.

[0094] Any probe or primer of at least 12 nucleotides in length derived from Bm3, Bm27, BS15, the amino DHQ synthase and the geldanamycin coding sequence (SEQ ID NO: 7) will be used to screen and select a strain useful as a biocontrol agent against common scab. In the alternative, a ligand to the protein geldanamycin may be used for the same purpose.

[0095] Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

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